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Abstract approved:

Reactive oxygen species (ROS) are necessary for normal development; however, ROS may cause damage to a developing fetus if present in excessive amounts, as can be the case in maternal drug use or maternal disease states. Cyclophosphamide (CP) is a complex multifaceted teratogen with mechanisms of teratogenesis thought to include production of excessive ROS. N-acetyl-L-cysteine (NAC) is a powerful antioxidant that offers protection from the toxicity of certain anticancer drugs, like doxorubicin and CP. No studies have explored the potential of NAC to attenuate CP-induced damage to the conceptus. The current study explored the effect of concurrent exposure to NAC and CP. Mated CD-1 mice were orally dosed with 150 mg/kg/d NAC, 150 mg/kg/d NAC + 20 mg/kg CP, CP only, or vehicle only. CP was administered by intraperitoneal injection on gestation day (GD) 10 and NAC was given by gavage on gestation days (GD) 6-13. Dams were sacrificed on GD 17, and their litters were examined for adverse effects. There was a significant reduction in the incidence of digit, limb, and tail defects, as well as anasarca and macroglossia in fetuses exposed to the combination of NAC and CP compared to fetuses exposed to CP only. NAC did not increase the incidence of any defects when compared to control. Fetuses exposed to NAC, on average, weighed slightly, though not significantly, more than the average control fetus. The data indicate that NAC is a well-tolerated, relatively inexpensive antioxidant that appears to reduce the

incidence of specific cyclophosphamide-induced malformations when administered prior to, concurrently and after exposure to CP.

Keywords: cyclophosphamide, teratogen, antioxidants, N-acetyl-L-cysteine, reactive oxygen species

EVALUATION OF EFFECTS OF EXPOSURE TO N-ACETYL-L-CYSTEINE ON CYCLOPHOSPHAMIDE TERATOGENESIS IN CD-1 MICE

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PREFACE

This thesis was prepared following the publication style of Birth Defects Research and was conducted in accordance with the Emporia State University Animal Care and Use Committee (Approval number: ESU-ACUC-14-001).

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I. INTRODUCTION

Excessive oxidative stress is a concern during pregnancy, and physiological abnormalities leading to *in utero* death or birth defects induced by reactive oxygen species (ROS) are well known. Exposure to excessive amounts of oxidative stress can be caused from maternal treatment with drugs such as cyclophosphamide (CP) or from a maternal disease state like diabetes (Dicke, 1989; Wells et al., 2009).

Cyclophosphamide (CP) is widely used to treat neoplastic and autoimmune diseases, including lymphoma, leukemias, lupus, multiple sclerosis, myasthenia gravis, scleroderma, and rheumatoid arthritis (Ayash et al., 1992). It is also one of the best known and well-studied teratogens, causing a variety of birth defects, mainly central nervous system and skeletal abnormalities, in the fetuses of pregnant animals treated with the drug at dosages that are not maternally toxic (Latorre et al., 2007; Mirkes, 1985).

By itself, CP causes neither neoplastic nor teratogenic effects (Hales, 1979). Cyclophosphamide is rapidly absorbed, but it is a prodrug and therefore needs to be bioactivated to achieve neoplastic and teratogenic effects (Hales, 1980). CP bioactivation is mediated by cytochrome P-450 enzymes, specifically CYP3A4 and CYP2B6, in the liver, to produce hydroxycyclophosphamide and in turn, its tautomer aldophosphamide (Klaassen, 2008). The final fate of CP is the outcome of two possible pathways, one being enzymatic and the other non-enzymatic (Mirkes, 1985). The enzymatic pathway is considered the route of detoxification and produces inactive metabolites with very little biological activity (Struck, 1971). The non-enzymatic pathway causes spontaneous breakdown of aldophosphamide to form phosphoramide mustard and acrolein. Phosphoramide mustard is responsible for the antineoplastic effect through DNA

alkylation and the acrolein is thought to be the cause of some adverse side effects of CP chemotherapy, like cystitis (Mirkes, 1985).

ROS are produced through this breakdown of CP into its metabolites. In controlled quantities, ROS are not typically problematic, and, in fact, ROS are used as signaling molecules. These signaling molecules are a key influence in signaling pathways involved in proliferation, differentiation, and cellular fate for normal development (Wells et al., 2009). In excess, however, ROS cause an imbalance between pro- and anti-oxidative species, leading to a condition known as oxidative stress. During oxidative stress, cells accumulate ROS from the process of making energy (ATP), detoxifying drugs, and from environmental sources. ROS have the ability to bind covalently to DNA, protein, and lipid structures. This process of binding is called oxidative stress, which alters cellular function and can result in *in utero* death (Wells and Winn, 1996). The teratogenic effect of CP comes at least partially from its ability to induce oxidative stress within the system (Hansen, 2006).

The adverse effects caused by excessive ROS can be balanced with antioxidants, which are effective *in vitro* for preventing conditions associated with oxidative damage through radical scavenging (Frei and Higdon, 2003; Halliwell, 1996; Wells et al., 2009). Antioxidants work mainly by donating an electron to stabilize ROS (Fliege, 2000). Some of these antioxidants are produced in the body, like glutathione and melatonin, while many others, such as vitamin C and E are obtained through dietary supplements or food (Chu et at., 2007). Exposure to antioxidants, like N-acetyl-L-cysteine (NAC), may reduce the incidence and severity of birth defects induced by excessive oxidative stress.

NAC is a thiol-containing cysteine derivative that was introduced as a mucolytic agent in the 1960s (De Flora et al., 2001). This well-known thiol antioxidant can function as both a redox buffer and a free-radical scavenger against endogenous free radicals or xenobiotics, both *in vitro* and *in vivo* (Erkkila et al., 1998; Meyer et al., 1994). NAC offers protection from the toxicity of certain anticancer drugs, like doxorubicin and CP (De Flora et al., 2001). Following its uptake, NAC is deacetylated to yield L-cysteine, which stimulates intracellular glutathione (GSH) production (Erkkila et al., 1998). GSH, a tripeptide made up of glutamic acid, glycine, and cysteine, plays a key role in protecting cells against toxicants, oxidants, and DNA damaging agents (De Flora et al., 2001; Dekhuijzen, 2004). NAC also shows nucleophilic properties, which allow it to combat free radicals through the processes of conjugation and reduction (Ornaghi et al., 1992).

Although studies examining the interaction between NAC and CP have been performed, no published studies to date have addressed the effects of NAC on CP teratogenesis in developing mice. This project examined the effects of NAC on the development of CD-1 mice, using cyclophosphamide to induce birth defects. Given the antioxidant properties of NAC, it is not unreasonable to believe that NAC will reduce the negative effects induced by cyclophosphamide in developing CD-1 mice, by increasing fetal weight and decreasing the percentage of gross and skeletal defects in the fetuses.

II. MATERIALS AND METHODS

ANIMALS AND HUSBANDRY. I purchased 135 female and 25 male CD-1 mice from Harlan Laboratories (Indianapolis, IN) and housed at Emporia State University's USDA-approved animal facility. The animal facility is maintained at 22±2º C at 50-70% humidity with a 12-hour light/dark cycle. Mice were acclimated for a minimum of two weeks prior to mating. During that period, mice were gang housed in single-sex cages. Mice received Teklad LM-485 rodent chow from Harlan Laboratories (Madison, WI) and tap water *ad libitum*.

After the acclimation period, each mouse was uniquely identified by ear punch. The mice were bred naturally, two females to one male. Females were examined for evidence of a copulation plug three times (8am, noon, and 8pm) the following day and every day thereafter for one week. Gestation day (GD) 0 was defined as the day a copulation plug was found. Mated females were housed individually and randomly assigned to a treatment group. All procedures performed on mice were in accordance with established guidelines set by Emporia State University's Institutional Animal Care and Use Committee (permit #ESU-ACUC-14-001)

TEST CHEMICALS. All chemicals were purchased from Sigma-Aldrich (St. Louis, MO). NAC solution was prepared by dissolving 0.15 g of NAC (150 mg/kg) in 10 mL of deionized (DI) H_2O. Cyclophosphamide solution was prepared by dissolving 0.02 g of CP in 10 mL of normal saline solution. Every solution was prepared freshly on the day that it was administered.

TREATMENTS. Females were randomly assigned to one of the four treatment groups: vehicle control (DI H_20); NAC 150 mg/kg/d; NAC 150 mg/kg/d + CP 20

mg/kg/d; or CP 20 mg/kg/d. Dams were weighed on GD 0 and prior to every dosing. NAC or DI H_20 was administered by gavage from GD 6-13. CP or saline was administered by intraperitoneal injection on GD 10.

DATA COLLECTION. On GD 17, females were euthanized via CO_2 overdose, the uteri exposed, and the number of resorptions and dead or live fetuses were recorded. Each of the live fetuses were removed from the uterus, weighed, and examined for gross malformations. Maternal body weight minus the weight of the gravid uterus was then recorded to determine if there was a difference in maternal weight gain among the treatment groups and control group. Fetuses were initially fixed in 70% ethanol for preservation and then cleared and stained by the double staining technique described by Webb and Byrd (1994).

DATA ANALYSIS. The litter was used as the experimental unit for statistical analyses. Mean fetal weight, mean maternal weight gain, and the incidence (as percentage of affected fetuses) of each defect were calculated. Gross defects were grouped for analysis as follows: head (exencephaly and exencephalocele), digit (polydactyly, oligodactyly, syndactyly, brachydactyly and combinations thereof), limb (meromelia, phocomelia, and talipies), tail (short or bent tail), macroglossia, anasarca, and ablepharia. Incidence of defects per litter, maternal weight gain, and mean fetal weight were analyzed by one-way analysis of variance (ANOVA), followed by an LSD (least significant difference) post hoc test to determine the specific difference among groups ($p \leq 0.05$). Both ANOVA and LSD post hoc tests were calculated using the Statistical Package for the Social Sciences (SPSS), version 14.0 for Windows (SPSS Inc., Chicago, IL).

III. RESULTS

MATERNAL DATA. There was no significant difference in maternal weight gain among the treatment groups (Table 1). No clinical signs of maternal toxicity, such as lethargy, ataxia, ocular discharge, nasal discharge, abnormal respiration, or piloerection, were noted among any of the treatment groups.

FETAL DATA. The number of resorbed or dead fetuses was not significantly different among any of the groups. Fetal weight in the NAC only group was significantly higher than controls ($p = 0.024$). Exposure to CP, either alone or in combination with NAC, significantly reduced fetal weight compared to controls ($p \leq 0.01$). Administration of NAC did cause a small recovery in fetal weight compared to fetuses exposed to CP only, but the difference was not significant ($p = 0.060$) (Table 1). The percentage of fetuses displaying any type of gross malformations was not significantly different between the control group and those exposed to NAC only. The incidence of digit, limb, and tail defects were significantly reduced in the NAC + CP group compared to the CP group ($p \leq 0.01$). Anasarca and macroglossia were also significantly reduced in fetuses exposed to the combination of NAC and CP compared to fetuses exposed to CP only ($p \leq 0.01$). There were no significant differences in head defects or ablepharia between combined NAC + CP and CP only groups (Figure 1).

The incidence of skeletal abnormalities, as with gross malformations, was not significantly different between the controls and the NAC treatment group. The percentages of fetuses with rib (rudimentary or supernumerary) and vertebral (dumbbell or fused centra) defects were significantly reduced in fetuses exposed to NAC and CP than in fetuses exposed to CP alone ($p \leq 0.05$). No statistical difference was seen in other

vertebral (notched cervical vertebrae) and rib defects (ossification spots), or sternum bipartite (Figure 2).

IV. DISCUSSION

NAC, a cysteine derivative, is an effective chemopreventative agent and has shown many cancer protective effects (De Vries and De Flora, 1993; Van Schooten et al., 2005). The objective of this study was to examine the potential of NAC as a protective agent against a well-known proteratogen, CP. The mechanism behind its teratogenic properties is not completely understood, but it is thought to be caused, at least in part, by the ability of CP to induce oxidative stress by flooding the system with ROS (Hansen, 2006). One way this damage can be balanced is through the use of antioxidants (Wells et al., 2009).

CP is a prodrug and therefore has to be bioactivated before it can cause neoplastic or teratogenic effects (Hales, 1979). This bioactivation is performed by p450 enzymes in the liver during phase I metabolism (Klaassen, 2008). During phase II metabolism CP is detoxified (Klaassen, 2008). Transferase enzymes are responsible for phase II reactions, namely glutathione-S-transferase (GST). GST reduces the ability of ROS to react with nucleic acids and proteins by promoting conjugation of ROS to GSH. NAC is a precursor to GSH. NAC becomes deacetylated to form l-cysteine, which supports biosynthesis of GSH. When GSH levels are increased, so are GST levels. NAC not only increases the amount of GST, but has been shown to enhance its activity as well (Moradi et al., 2009). Thus, NAC's influence on phase II metabolism may be one of the likely mechanisms for reducing the teratogenic effects of CP observed in this study.

NAC is a well-known thiol antioxidant that is very efficient as a redox buffer, as well as, a free radical scavenger (Erkkila et al., 1998). Thiol groups are essential in the defense against ROS (De Vries and De Flora, 1993). NAC is thought to work by two

possible mechanisms. As discussed above, NAC is a precursor to GSH. (Klaassen, 2008). NAC also has the ability to act as a strong nucleophile, attacking oxidant radicals directly. The nucleophilic and reducing properties of NAC alter mutagenicity of direct acting compounds such as epichlorohydrin, sodium dichromate, and hydrogen peroxide. NAC protects DNA from promutagens by acting as a nucleophile and by lessening mutagen induced chromatid breakage. NAC also regulates DNA repair after damage has already been done by protecting nuclear enzymes and correcting DNA methylation (De Vries and De Flora, 1993; De Flora et al., 2001).

These findings are comparable to many other studies that examined the beneficial effects of NAC. Botta et al. (1973) studied a number of agents in an effort to prevent cyclophosphamide-induced cystitis. Of all the agents in their study, N-acetyl-l-cysteine was the most effective. Two mechanisms were proposed for its protective effects. One was that NAC reduced inflammation in the bladder; a later study, by Moradi et al. (2009) found that NAC reduced the formation of proinflammatory cytokines such as interlukin-8 and tumor necrosis factor (Moradi et al., 2009). The other proposed mechanism was that NAC was inactivating alkylating metabolites of CP (Botta et al., 1973). The notion that NAC acts at least in part by neutralizing free radicals was further supported, when Doroshow et al. (1981) examined the effect of NAC on doxorubicin toxicity in mice. NAC was effective in blocking cardiac toxicity, but did not affect uptake or metabolism of doxorubicin in the spleen or the liver. One proposed reason as to why it did not affect uptake or metabolism is because doxorubicin's major cytotoxic effect on tumor cells is not related to the formation of free radicals.

The dosage of NAC (150 mg/kg/d) that the dams were exposed to in this study is considered pharmacological compared to what a typical dietary supplement for human consumption would be. A person using NAC as a dietary supplement would be in the form of 600 mg capsules and the recommended daily dose is two capsules. This means that a typical dose of NAC would be about 17 mg/kg for an average 70 kg adult.

The exact mechanism for the results observed in this study remain unclear; however, previous literature strongly suggests that NAC supports phase II biotransformation by causing an increase in GST, by directly scavenging and neutralizing free radicals created by CP, or by some combination of the two. The data from this study show that exposure of NAC, at pharmacological dosages, prior to, concurrent, and after CP exposure can reduce the incidence and severity of birth defects caused by CP exposure and that NAC itself does not cause materno- or embryotoxicity. The implications for human pregnancies cannot be directly extrapolated from the results of this study; however, its use may be an avenue worth pursuing given the affordability, ease of use, and popularity of NAC as a dietary supplement.

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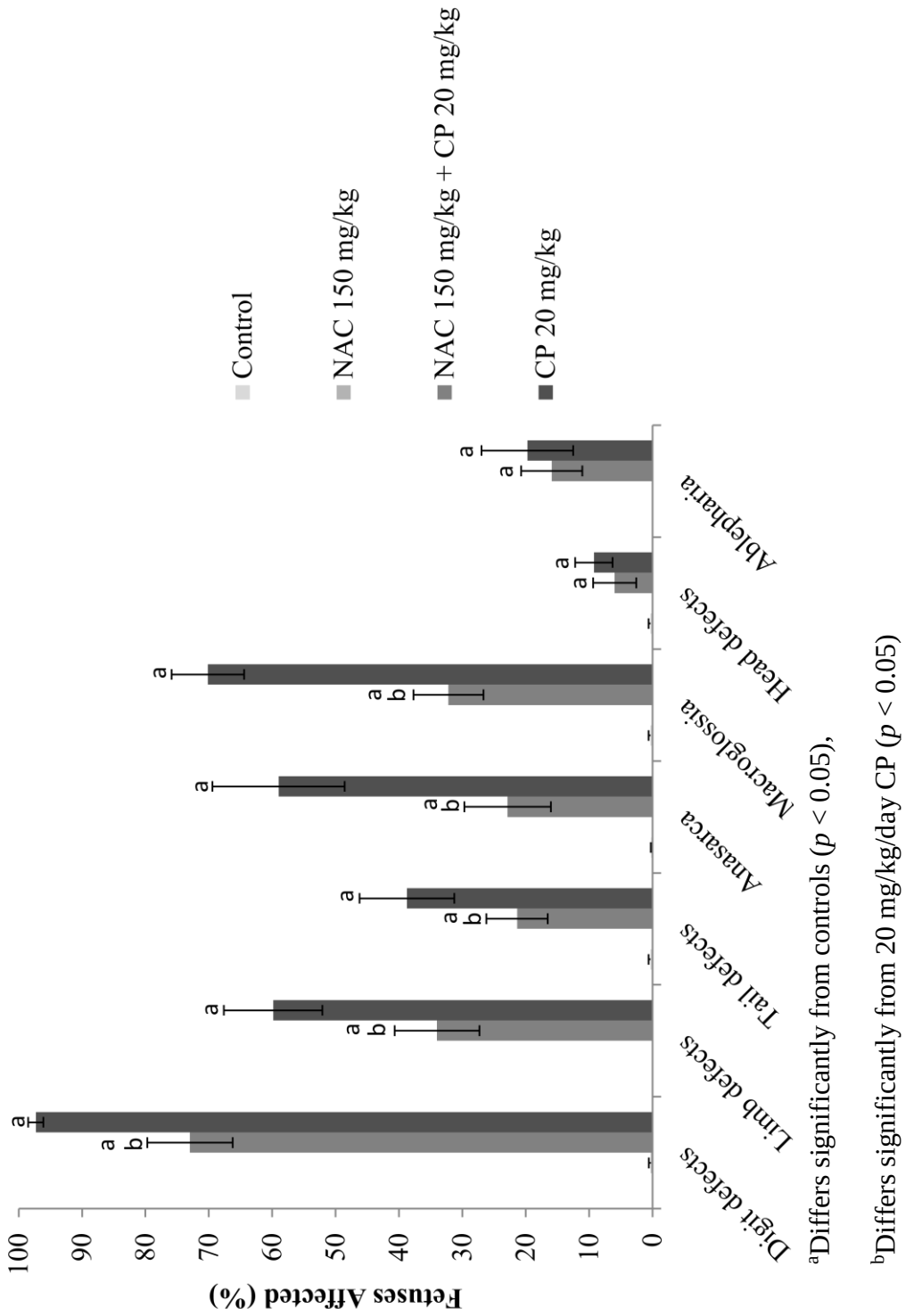
TABLE 1

Maternal and Litter Parameters of Mice Treated During Gestation With Cyclophosphamide (CP) With or Without Exposure to N-acetyl-L-cysteine (NAC)

	Treatment and Dose (mg/kg/day)			
	Vehicle Control	NAC 150	NAC 150 + CP 20	CP 20
Litters examined (Fetuses/Litters)	349/25	353/25	378/29	173/22
Maternal weight gain (g ± SEM)	12.98 ± 0.58	14.21 ± 0.54	12.62 ± 0.59	13.44 ± 0.47
Fetal weight (g ± SEM)	1.00 ± 0.02	1.07 ± 0.02[a]	0.71 ± 0.02[a]	0.65 ± 0.02[a]
Implantations (mean ± SEM)	14.24 ± 0.39	14.28 ± 0.45	13.70 ± 0.37	13.71 ± 0.37
Resorbed dead fetuses (% ± SEM)	1.60 ± 0.59	1.08 ± 0.63	5.13 ± 3.17	9.38 ± 4.84
Litters with resorbed or dead fetuses (No./%)	5/20	3/12	6/21	7/32

[a]Differs significantly from controls ($p < 0.05$),

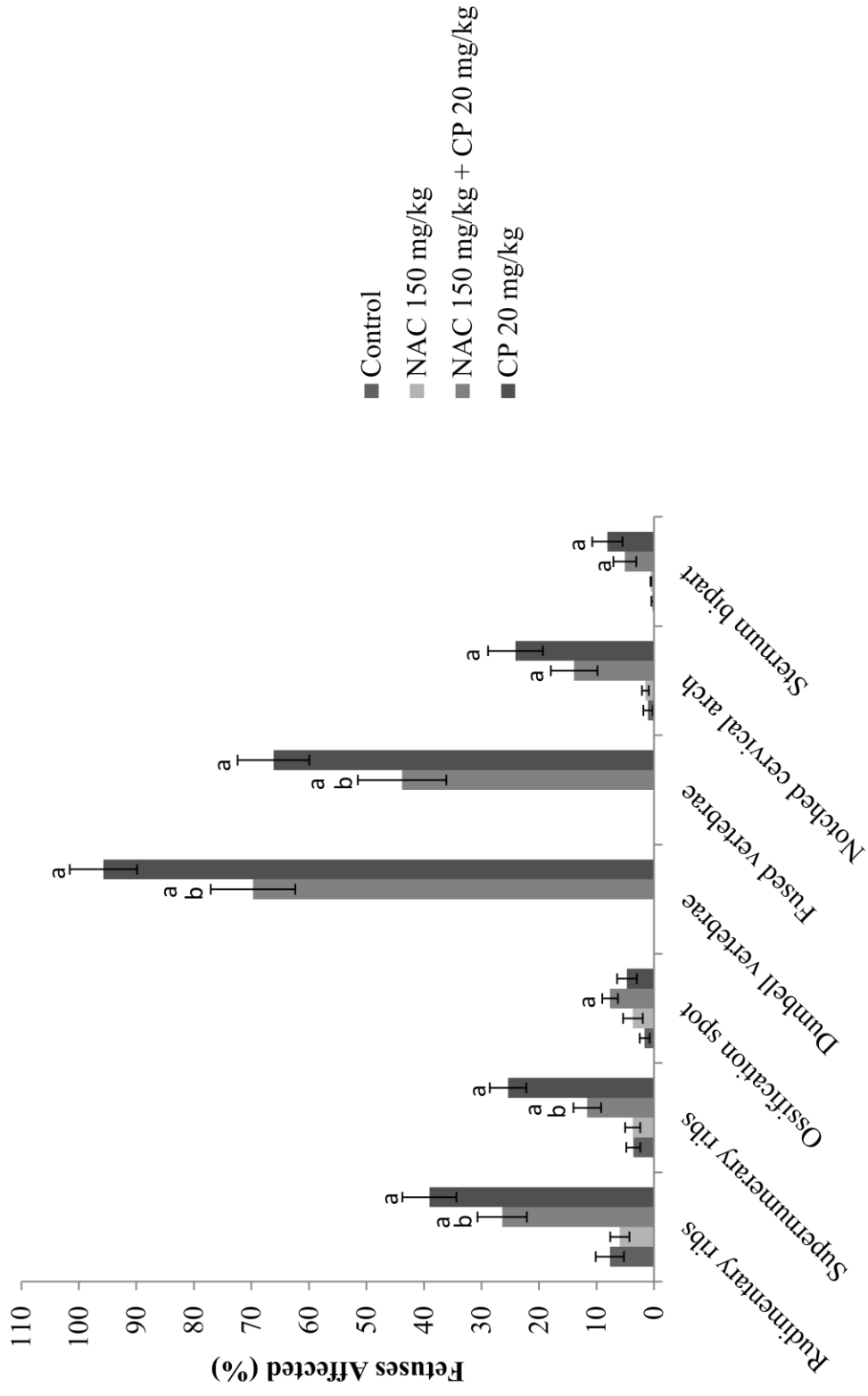
FIGURE 1---Incidence of Gross Malformations in Mouse Fetuses Following Maternal Exposure to Cyclophosphamide (CP) During Gestation, With or Without Exposure to N-acetyl-L-cysteine (NAC)



[a]Differs significantly from controls ($p < 0.05$),

[b]Differs significantly from 20 mg/kg/day CP ($p < 0.05$)

FIGURE 2---. Incidence of Skeletal Malformations in Mouse Fetuses Following Maternal Exposure to Cyclophosphamide (CP) During Gestation, With or Without Exposure to N-acetyl-L-cysteine (NAC)



[a]Differs significantly from controls ($p < 0.05$) and

[b]Differs significantly from 20 mg/kg/day CP ($p < 0.05$)

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